EXHIBIT G

High-throughput proteomics using antibody microarrays

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Antibody-based microarrays are a novel technology that hold great promise in proteomics. Microarrays can be printed with thousands of recombinant antibodies carrying the desired specificities, the biologic sample (e.g., an entire proteome) and any specifically bound analytes detected. The microarray patterns that are generated can then be converted into proteomic maps, or molecular fingerprints, revealing the composition of the proteome. Using this tool, global proteome analysis and protein expression profiling will thus provide new opportunities for biomarker discovery, drug target identification and disease diagnostics, as well as providing insights into disease biology. Intense work is currently underway to develop this novel technology platform into the high-throughput proteomic tool required by the research community.

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New and promising avenues for biomedical research are rapidly emerging in the post-genomic era. The availability of whole genomes from hundreds of species has generated high expectations for curing many diseases. Sequencing of the human genome revealed approximately 30,000 genes that are believed to encode more than a million proteins with distinct functional properties. Aberrant expression and function of any of these proteins in the proteome can result in disease. This is information that cannot be obtained by gene expression analysis alone. Proteomics and global proteome analysis will thus play a key role in the identification, characterization and screening of all proteins [1].

In analogy to DNA microarray technology [2], protein microarrays outline a possibility to develop rapid global analysis of the entire proteome (FIGURE 1) [3]. Two general types of protein microarrays have been developed [3]. First, functional protein microarrays, in which a set of proteins are arrayed and evaluated for biochemical activities (e.g., enzymatic activity and functional proteomics) [4,66]. Second, affinity microarrays, in which specific binders, such as antibodies, are used to detect and quantitate protein analytes (quantitative proteomics) (FIGURE 1) [5]. The latter group of microarrays has generated high expectations and will play a significant role within the field of proteomics [3,5–9]. It will also help supplement current technologies such as 2D gels and mass spectrometry (MS). The array patterns generated can be converted into detailed proteomic maps revealing the composition of the proteome. The technology will permit comparative proteome analyses to be performed on any sample format in a species-independent manner as long as a proteome can be isolated. The concept of comparing proteomic maps of healthy with diseased samples may allow scientists to address areas such as [3,6–9]:

- Signaling and metabolic pathways to identify disease-related proteins
- Examine protein–protein interactions of functional networks
- Perform differential protein expression profiling, disease diagnostics and biomarker discovery
- Form a novel base for drug development

Furthermore, antibody-based affinity microarrays can be used for both focused and global quantitative proteomics [3,5–9]. In fact, antibody arrays have the potential to revolutionize the way we analyze and examine several diseases, such as cancer. By simultaneously monitoring the expression of numerous proteins in clinical specimens, a wealth of data is generated that

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coalesce to form a molecular fingerprint of the disease at the protein level. Detecting and/or monitoring the levels of tumor markers may aid in diagnosis, population screening, assessing responses to treatment, and in developing new therapeutics. Therefore, antibody-based microarrays are an emerging class of proteomic technologies and major efforts are currently underway to develop this approach. In the last few years, the first applications in which antibody arrays have been used for protein expression profiling and limited proteomic profiling have been published [10–13]. In these examples, low- to medium-density arrays were used for multiplex analysis of complex samples (e.g., cell lysates and serum) targeting mainly water-soluble analytes [10–13]. Recently, the possibility of targeting membrane proteins using antibody arrays was outlined [14,15].

Proteome analysis: clinical relevance & current status

The impact of proteome analysis in medicine is substantial, a feature that will grow in importance over the coming years [1,16,17]. Several studies have been published in which proteomic approaches have been used in various disease-related applications, ranging from the identification of changes in protein expression to biomarker discovery and drug target identification [1,16,17]. In this context, major focus has been placed upon the human plasma (serum) proteome due to the central role plasma plays in disease diagnosis and biomarker discovery [18–21]. However, the properties of the human plasma proteome illustrate some of the formidable challenges facing disease proteomics. The complexity of human plasma is substantial as it is estimated to contain up to 10,000 different proteins at a dynamic range of at least nine orders of magnitude [19–21]. Furthermore, approximately 99% of the protein content is made up of only 22 proteins [18]. Most plasma proteins, including several classes of physiologically relevant proteins, such as cytokines and chemokines, will be present at very low concentrations. However, it is also among this group of low-abundance proteins that most novel biomarkers are expected to be identified. To some extent, these issues have been met with improved sample preparation techniques involving prefractionation and/or protein depletion methodologies to reduce sample complexity [16,19]. However,

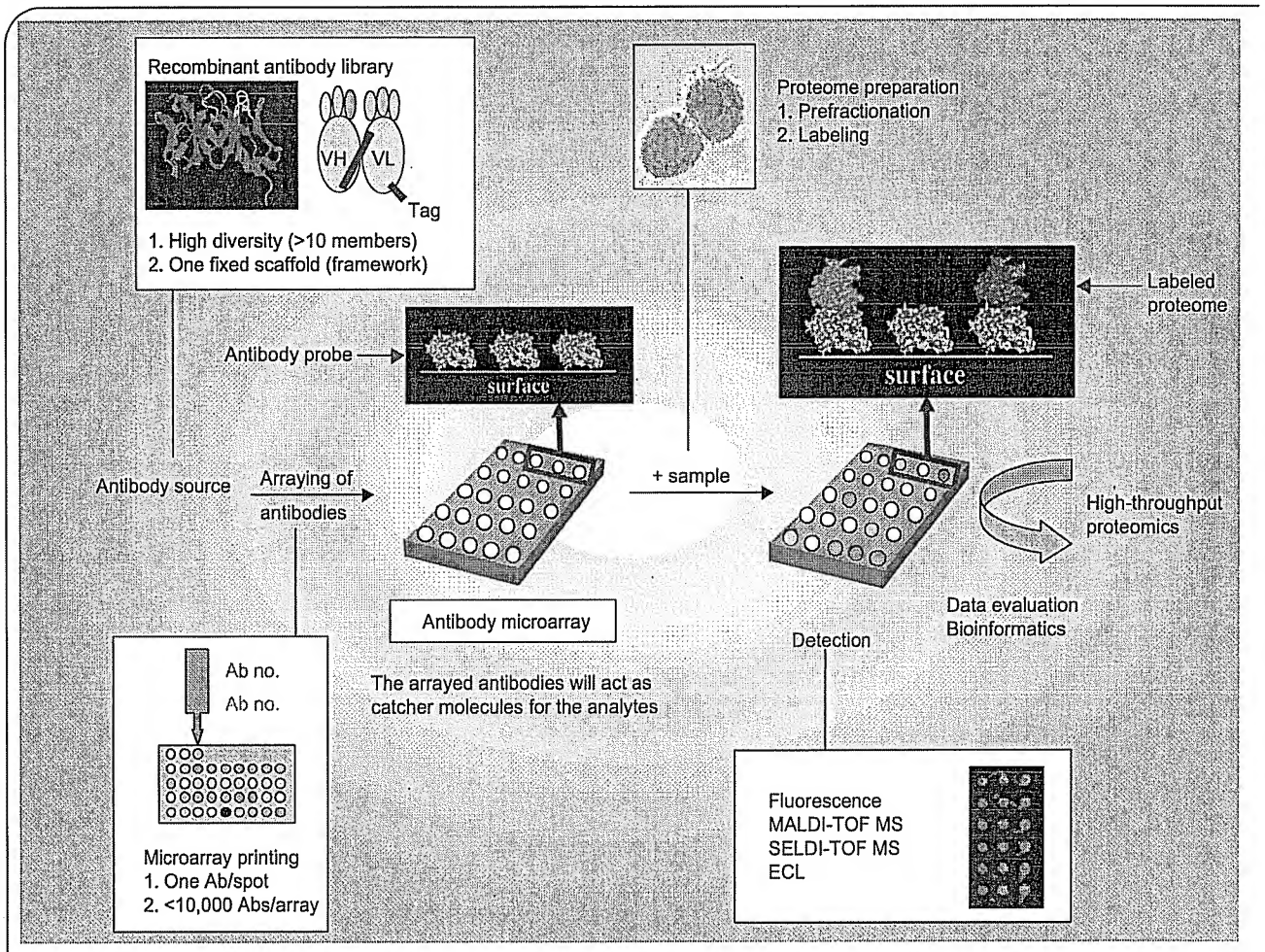

Figure 1. Schematic illustration of the antibody microarray concept.
Ab: Antibody; ECL: Electrogenerated chemiluminescence; MALDI: Matrix-assisted laser desorption/ionization; MS: Mass spectrometry; SELDI: Surface-enhanced laser desorption/ionization; TOF: Time-of-flight; VH: Very heavy; VL: Very light.

the demands for highly multiplex, high-throughput proteomic approaches displaying high specificity/selectivity and sensitivity are considerable.

In traditional proteomics, various separation methods (e.g., 2D gels and multidimensional liquid chromatography) coupled with MS have evolved into a commonly used versatile tool [16–21]. However, as the number of proteomic projects and high-throughput methods have exploded during the last few years, it has become clear that no single technology will meet the needs of all proteomics-based investigations [16,17]. Integration of different data sets generated by multiple strategies and technology platforms will undoubtedly be required [16,17]. Antibody-based microarrays will provide one such technology platform. This review will discuss:

- The current status of antibody microarray technology
- Key issues in the process of evolving this technology into a proteomic research tool
- Present and future applications

Antibody microarray technology

Probe design

The choice of probe is a key issue in the process of designing affinity microarrays for proteome analysis [3,5,7,10]. To date, antibodies [5,10], or various antibody mimics based on other scaffolds (e.g., affibodies [101], aptamers [102], molecule imprints [103], ribozymes [104] and trinectin-binding proteins [105]), have been evaluated as potential capture agents. However, the probes must be easy to handle, renewable and give rise to dense arrays displaying high functionality, stability, specificity, diversity and sensitivity, which directly rules out some of the aforementioned probes. Furthermore, using probes based on the same molecular scaffold (i.e., as identical as possible) will minimize assay variations due to individual molecular properties. Currently, antibodies have emerged as the primary candidate for sensor molecules, since recombinant antibody libraries fulfill most, if not all, of the required criteria [5,6,10]. Of note, antibodies can display an exquisite specificity and can even distinguish between (single) functional groups on proteins, a property not easily matched by any other groups of proteins, whether involved *in vivo* or *in vitro*. Despite this feature, several recent studies have raised serious concerns as to whether antibodies are specific enough [3,17,22–24]. One likely explanation is that readily available on-the-shelf antibody reagents were mainly used directly as content. The approach of using such reagents is problematic, as these antibodies have not been designed and selected for the properties that they are expected to display in antibody microarrays [10,17]. For example, early work demonstrated that less then 20% of arrayed monoclonal antibodies displayed adequate reactivity [25]. In more recent work, it has been indicated that only 5% of over 100 commercially available monoclonal antibodies tested were suitable for microarray-based analysis [3].

The first generation of antibody-based microarrays were generated as content mainly using monoclonal and occasionally polyclonal antibodies [3,25,26]. Despite the success in using these probe designs, several limitations were also identified [3,27]. First, the number of available monoclonal antibodies is small, thus finding existing antibodies with the desired specificities will be a limiting factor. Second, the issue of scaling up the arrays is a major bottleneck, since producing monoclonal antibodies against thousands of antigens will be overwhelming. Third, monoclonal antibodies will display a wide range of molecular properties (e.g., on-chip stability), making them well suited as differential probes.

However, these limitations can be eliminated, or at least minimized, by using recombinant antibody libraries as the probe source [5,10,28,29]. Large ($>10^{10}$ members) human recombinant single-chain (sc)Fv [30,31] and Fab antibody phage display libraries are now available [106,107,116]. These highly diverse libraries can provide an almost limitless number of probe molecules, based on a single molecular scaffold displaying high functionality, specificity and sensitivity [30,106,107,116]. Furthermore, the fixed scaffold can be designed with superior molecular properties (e.g., probe stability [28]), including carrying the desired affinity tag(s) for optimal purification and/or coupling of the probes. The best molecular properties can then be directly incorporated into all members in the library in a single step procedure. This is a major advantage compared with using monoclonal antibodies as probes, since the design of these molecules would have to be optimized individually.

When designing an antibody library for microarray applications, the precise choice of scaffold will therefore be essential as it will govern the molecular properties displayed by the probes [28,32]. For example, the half-life of arrayed dehydrated scFv molecules based on different frameworks has been found to range from a few days up to several months [28]. To date, the best performing scFvs have been found to display a functional on-chip stability (80–100% retained antigen-binding activity) at room temperature exceeding 7 months [STEINHAUER ET AL, UNPUBLISHED OBSERVATIONS]. Ultimately, such an impressive probe performance will be a prerequisite in designing highly functional antibody-based microarrays for high-throughput proteomics.

Substrate design

Design of the solid support is also essential in the process of developing antibody-based microarrays or protein arrays [33–35]. Currently, arrays have been produced predominantly on glass, plastic or silicon slides, usually coated with 1-, 2- or 3D structured surface modifications [33–37,108–113]. Four key properties of the solid supports are:

- High biocompatability
- High and selective probe-binding capacity
- Ability to bind the probes in a favorable orientation
- Low nonspecific binding (background)

High biocompatability is essential as arraying protein-based probes onto solid supports often results in partial or complete denaturation and subsequent loss of biologic activity [33]. Silicon-based substrates [37] and glass surfaces modified with nitrocellulose [38,39,111] or various hydrogels [40,41,108] are some

examples of promising supports [33]. In the case of hydrogels, minute amounts of water may be trapped within the 3D structure of the substrate providing a semimoist environment, appropriate for protein arrays [42]. However, this issue can be addressed to some extent by selecting and engineering probes to survive demanding conditions. The probe binding capacity, an important feature for assay sensitivity, can be improved by surface structure engineering [33]. The transition from 1D supports (e.g., poly-L-lysine-coated glass slides) and 2D designs (e.g., polyethylene glycol-modified surfaces) to 3D substrates (e.g., macroporous silicon) can increase the amount of probe that can be bound per surface area. The selectivity of the probe-binding process and the orientation of the bound molecules are determined by the choice of probe coupling chemistry. Three major binding approaches are available: adsorption, covalent binding and affinity binding [33]. Physical adsorption is the simplest approach that appears to work well in most cases. As the approach is nonselective (any irrelevant proteins present in the probe solution can be coadsorbed) and the probes are randomly orientated and reversibly bound, the density of functionally adsorbed probes can be low. In the case of covalent binding, the probes are irreversibly bound. However, covalent coupling may impair the biologic activity of the probes and, as in the case of adsorption, the approach is nonselective and the probes are randomly orientated. Affinity binding on surfaces functionalized with affinity ligands, such as Ni^{2+} ions or streptavidin [4,43–46], will allow crude probe preparations to be purified, coupled, enriched and specifically orientated in a high-density one-step procedure directly on the chip (affinity-on-a-chip). This approach will require access to affinity-tagged probes, but most protein libraries are already designed to carry various tags that could be directly utilized. Nonspecific binding (high background signals) is a major problem, especially when analyzing complex samples, such as entire proteomes. The development of superior blocking techniques and/or inert supports will undoubtedly be of major importance.

Microarray design

The choice of array design depends on the application, ranging from small focused assays targeting a few analytes to global proteome analysis addressing thousands of analytes. Adopting current technologies, dense microarrays composed of less than 1000 probes may be fabricated. To date, mainly low- to medium-density antibody microarrays have been generated. The main reasons for the observed limitations in size are limited availability of numerous high quality probes and limited access to high quality substrates allowing for affinity binding. The fact that most, if not all, of the current substrates require prepurified probes poses a major logistic problem that must be resolved.

To be able to perform true global proteome analysis, microarray designs allowing greater than 10,000 analytes to be addressed simultaneously must be developed. In a recent publication, the design of a novel antibody array format, so-called megadense nanoarrays (>100,000 probes), was outlined [47]. It has been proposed that nanotechnology will provide us with the tools required to design and fabricate such nanoarrays [47]. Various methodologies are currently available (e.g., nanoimprint litography) that allow arrays displaying a density of up to 160 G-spots/cm^2 to be generated [48–50]. Recently, the first efforts to make addressable antibody nanoarrays produced on nanostructured surfaces was published [51]. Taken together, nanotechnology and nanoarrays will undoubtedly play a vital role within high-throughput proteomics.

Sample format

All samples generated in a soluble format can be analyzed by antibody-based microarrays. The reduced sample consumption in the microarray format is essential as often only minute volumes of precious samples are available; volumes in the picoliter scale may also be sufficient if the sample is arrayed using conventional spotters [29]. Sample complexity is a key feature that may impair analysis by making it difficult to label the samples in a representative manner, and/or by causing high nonspecific binding and thereby significantly reducing sensitivity of the assay.

To date, little attention has been placed upon the sample format within the field of antibody arrays. In traditional proteomics, various prefractionation strategies and/or removal of high-abundance proteins have been successfully applied in preparation for 2D gel analysis, which significantly improved the detection of low-abundance proteins [18–20]. However, one problem associated with any protein separation/depletion technique is that low-abundance proteins may be removed along with the high-abundance species [18,19]. In the case of antibody arrays, one recent study has shown that a simple one-step fractionation (based on size) of a proteome considerably enabled the detection of low molecular weight (<50 kDa) analytes in the sub pg/ml range [J INGVARSSON ET AL, UNPUBLISHED OBSERVATIONS]. The development of improved fractionation procedures will thus provide an avenue for further improving assay sensitivity.

Analytical principles & limit of detection

To perform adequately, antibody-based microarrays must have a limit of detection (LOD) in the picogram (attomole) range [27]. A theoretical detection limit of antibody-based microarrays of approximately 10 zM has been proposed [53]. In practice, a variety of analytical principles have been applied, resulting in a LOD in the zeptomole range or lower [13,25–27,29,39,54,55]. Thus, current technology platforms are already within the suggested range of LODs to be able to perform adequate proteome analysis.

Two major groups of analytical principles, based on either direct/indirect labeling or label-free detection technologies, have been applied as detection systems for antibody microarrays. In the former group, most arrays described thus far rely on fluorescence [3-15,29], and to some extent on chemiluminescence [52], as the read-out principle. The sample can be directly labeled with a fluorescent tag (e.g., Cy5) or a hapten (e.g., biotin). Analytes labeled with fluorescent dyes allow measurements with nonconfocal or confocal scanners

previously designed for reading DNA microarrays [108,113]. LODs in the nanomole to femtomole range have been reported [13,25,26,29]. In fact, a 300 zM analyte was sufficient for detection [29]. By adopting planar wave guide technology (fluorescence-based detection), a LOD of 2 pM corresponding to 0.8 zM (500 protein molecules) has been reported [54,121]. Recently, the detection limit for proteins in directly labeled complex samples, such as human serum, was proposed to be approximately 100 ng/ml [9]. Of note, recent data has indicated that a LOD in the subpg/ml range may indeed be accomplished for directly labeled serum samples using optimized single scaffold antibody arrays (e.g., scFv antibody arrays) [UNPUBLISHED OBSERVATIONS].

The LOD may be further improved (up to a 100 times) by adopting different signal amplification techniques, such as rolling circle DNA amplification (RCA) [22,55–57] or thyramide signaling amplification kits [108]. To circumvent the problems associated with direct labeling of the sample, sandwich assays can also be designed in which the primary reagent, instead of the sample, is labeled (i.e., indirect labeling) [27]. Furthermore, adopting a sandwich approach may also improve the specificity and sensitivity of the assay [27]. Assay LODs in the pM to fM range have been reported for such set-ups [38,54,55]. The sandwich approach works well, providing small focused arrays are constructed. However, as soon as the arrays need to be scaled up, such an approach is no longer viable, since the process of generating high quality sandwich antibody pairs against numerous analytes would be overwhelming. In addition, a threshold of approximately 50 probes per sandwich array has been proposed to maintain adequate assay features [9].

Within the field of protein microarrays as a whole, much interest exists in adopting label-free detection methods to eliminate all problems associated with protein labeling. As recently reviewed, several techniques, such as MS surface plasmon resonance (SPR), resonance light scattering (RLS) and surface-enhanced Raman scattering (SERS) microscopy, are currently being evaluated [5,7]. In the case of antibody-based microarrays, efforts have been made to incorporate matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS [56], surface-enhanced laser desorption/ionization (SELDI)-TOF MS [114], quart crystal microbalance with dissipation monitoring (QCM-D) [59], as well as nanomechanical cantilevers [60,61] as read-out systems. In these particular cases, LODs in the attomole range (sub-nM) – (MALDI-TOF MS) [58], femtomole range (SELDI-TOF-MS) [UNPUBLISHED OBSERVATIONS], picomole range (QCM-D) [59] and 20 µg/ml (cantilever) [60] have been observed. Although these approaches are promising, thus far they have only been used for small prospective arrays. Ultimately, analytical principles, such as MS and tandem MS, although time consuming, may allow the user to both detect and identify the bound analyte(s) in a one-step procedure directly on the chip. However, further developmental work will be required before any of these methods become generally available.

Antibody microarray applications

The first antibody microarrays were fabricated only 4 years ago [25,26]. Major efforts have since focused on developing the basic technology platform [3,5,7,8]. Although great progress has been made, the technology is still evolving and considerable work is in progress to further refine the method into the high-throughput proteomic research tool needed by the research community [3,5,7–9].

The number of applications in which antibody-based microarrays is used is anticipated to increase significantly within the next few years. To date, applications have been developed mainly within diagnostics, screening and protein expression profiling/focused proteome analysis. In the latter case, set-ups targeting soluble as well membrane-bound protein analytes have been designed [3,7–9,14]. In all cases, low- to medium-density antibody arrays (<500 probes) composed of predominantly intact monoclonal antibodies were used. Recently, the first microarray applications based on recombinant antibody fragments were also published [5,10,29].

Point-of-care applications

Small antibody array-like devices (<10 probes) have been designed for point-of-care applications, including diagnostics and screening of biologic samples [115]. A blood sample is added to the device and is automatically injected and read within 15 min. The device is based on a sandwich assay relying on fluorescence as the read-out system. Several different products for diagnostic use, such as detecting protein markers for acute myocardial infection and congestive heart failure, and for screening purposes, such as detecting the presence of commonly abused drugs (e.g., cocaine) or certain parasites (e.g., *Clostridium difficile*), are currently available [115].

Protein expression profiling

Dedicated arrays

Focused antibody microarrays against cytokines and chemokines were among the first dedicated arrays designed for (differential) protein expression profiling [10,56,62–64]. The early development of these arrays was strongly influenced by access to several commercially available monoclonal antibodies against cytokines, and sandwich pairs thereof, as well as by the potential commercial market for such arrays. Anticytokine chips are currently available from several different suppliers [108,111,117–120]. Huang and coworkers designed sandwich arrays for the specific detection of 24 cytokines, using electrogenerated chemiluminescence as the mode of detection [62]. The authors showed alterations in cytokine secretion from human glioblastoma cells upon treatment with tumor necrosis factor (TNF)-α [62]. Recently, a multiplex sandwich assay based on 51 anticytokine monoclonal antibodies and RCA as the read-out system was fabricated [56]. The authors demonstrated the sensitivity and specificity of the set-up for cytokine expression profiling in crude cell supernatants after stimulation of various agents, such as lipopolysaccharide versus TNF-α. Lin and coworkers also described the development and optimization

of anticytokine microarrays. However, in this study, directly biotin-labeled sample was applied [62]. The authors indicated the applicability of the set-up by profiling the cytokine expression in estrogen receptor (ER) positive and ER negative cells. In recent experiments, differential cytokine expression profiling was successfully performed on dendritic cells challenged with a proinflammatory cytokine cocktail, using recombinant scFv antibodies [J INGVARSSON, CAK BORREBAECK & C WINGREN, UNPUBLISHED OBSERVATIONS,10]. In these studies, directly labeled cell lysates and/or cell supernatants were analyzed on arrays based on ten to 84 scFv probes. The authors showed that specific upregulation of several cytokines could be detected, an observation that was further corroborated at both the gene and protein levels by matching DNA microarray analysis and enzyme-linked immunosorbent assay experiments [J INGVARSSON, CAK BORREBAECK & C WINGREN, UNPUBLISHED OBSERVATIONS,10].

Global perspective

For more general protein expression profiling of selected protein analytes involved in signal transduction, cell cycle regulation, gene transcription, apoptosis and oncogenesis, a commercially available microarray composed of approximately 500 monoclonal antibodies has been launched [122]. The microarray is designed to measure relative protein abundance. The set-up requires the sample to be directly labeled with fluorophores, using a two-color approach. The manufactures demonstrated that a variety of biologic samples, including soluble extracts from whole tissue, could be analyzed displaying sensitivity in the pg/ml range [122].

Zhou and coworkers applied two-color RCA on antibody microarrays for differential protein expression profiling of serum proteins [57]. The arrays were based on 35 monoclonal and polyclonal antibodies that were predominantly directed against highly abundant, common protein analytes. A total of 24 serum samples, from six liver cancer patients, six precirrhotic patients, six cirrhotic patients and six normal controls, were screened and the relative protein expression profiles were compared. The authors reported that patients with liver cancer and cirrhosis generally showed higher levels of most analytes compared with healthy controls [57]. However, the samples from similar disease indications did not cocluster, indicating that these proteins were not true markers for these particular disease states.

The serum proteome has also been addressed using recombinant scFv antibody microarrays [UNPUBLISHED OBSERVATIONS,10,29]. In these set-ups, the expression profiles of medium- (e.g., complement proteins) to low-abundant (e.g., cytokines) protein analytes were analyzed in directly labeled serum samples. Considering the complexity of human serum [19–21], these data clearly demonstrate proof of concept by recombinant scFv fragments, where the lack of cross reactivity illustrated the high specificity displayed by this content [10,29].

Cancer

The first reports demonstrating the applications of antibody-based microarrays to cancer research were recently published [11–13]. Sreekumar and coworkers applied antibody microarrays composed of 146 distinct probes to monitor alterations of protein levels in LoVo colon carcinoma cells treated with ionizing radiation [13,65]. Cell lysates were directly labeled with fluorophores in a two-color approach. The authors identified several proteins that were either up- or downregulated in the ultraviolet-treated samples [13]. Many of the upregulated proteins in the irradiated cells were regulators of apoptosis, an observation consistent with an increased frequency of apoptosis among these cells.

Knezevic and coworkers used antibody-based microarrays composed of 250 probes to analyze the protein expression profile in tissue derived from squamous cell carcinoma of the oral cavity [12]. The sample was directly labeled using a one-color approach. The authors reported differences in expression patterns of multiple proteins within epithelial cells that correlated with oral cavity tumor progression [12]. Similarly, differential expression of multiple proteins was also found in surrounding stromal cells that directly correlated with tumor progression of the epithelium. The antibody microarray results were confirmed by western blot and immunohistochemistry analysis.

Recently, Miller and coworkers reported the application of antibody microarrays for protein profiling of human prostate cancer versus normal sera [11]. Directly labeled serum samples from 33 prostate cancer patients and 20 controls were analyzed on arrays composed of 184 monoclonal or polyclonal antibodies. The authors identified five serum proteins that had significantly different levels between the cancer samples and the controls [11]. Four of these proteins had previously been associated with prostate cancer. Taken together, these experiments clearly demonstrated the potential of antibody microarrays to detect serum biomarkers.

Membrane protein profiling

To generate correct and complete maps of the entire proteome, both water-soluble and membrane proteins must be addressed. Membrane proteins constitute an extremely important group of proteins. They are one of the most common targets for disease diagnostics, biomarker discovery and therapeutic antibodies. However, they are often considered as a difficult group of proteins to analyze. Recently, Belov and coworkers reported the successful application of antibody-based microarrays for immunophenotyping of leukemias by targeting membrane-bound cell surface proteins of intact cells [14,15]. To date, arrays composed of 60–90 distinct antibodies directed against defined cluster of differentiation (CD) antigens have been fabricated. The authors demonstrated a rapid and concurrent screening of leukocyte suspensions for expression of multiple CD antigens [14,15]. Ultimately, multiplex immunophentyping of leukemias and lymphomas using antibody microarrays can be applied for disease diagnostics as well as for basic research in cell biology and immunology. Our preliminary experiments have also outlined the possibility of using recombinant scFv antibody microarrays for profiling membrane protein antigens [DEXLIN ET AL, UNPUBLISHED OBSERVATIONS].

Summary & conclusions

Antibody-based microarrays are a novel technology that holds great promise within proteomics-based investigations. The technology has evolved significantly during the last few years, where several critical steps have been taken, especially in probe design, which is the crucial point in all antibody array work. Major efforts have also been made to develop and further refine the basic methodology with respect to surface design, array design and analytical principles. As a result of these efforts, several high-performance antibody microarray technology platforms have emerged, upon which the first proteomic applications have been based. Current platforms have already proved capable of performing rapid and highly multiplexed (focused) proteome analysis targeting low-abundant protein analytes. However, it should be noted that the full potential of the technology will only be exploited when remaining key issues, such as scaling up the arrays, have been solved. Still, it is clear that antibody-based microarrays belong to the emerging class of novel proteomic technologies that, in the near future, have the potential to display a true high-throughput format.

Expert opinion

Antibody microarrays have the potential to mirror previous breakthroughs in genomics that were enabled by high-density DNA microarrays developed during the last few years, but at the protein level. From a biomedical point of view there is a tremendous interest to perform high-throughput proteomic analysis since most pharmaceuticals are targeting proteins and not genes. Furthermore, the potential to study disease development and progression, signaling pathways, cell differentiation and gene regulation is almost limitless using antibody microarrays, since the amount of information obtained in a very short period of time is impressive. What remains before we can call this a reality?

First, the density of antibody microarrays must be increased to more than 10,000 and perhaps to even 100,000 probes/chip, since global proteomic fingerprints will require a much higher density than can be produced today. The analogy to developments in genomic analysis is obvious and will take us into nanotechnology approaches where 10^6 points/mm^2 can be realized today [47]. To reach this density, problems surrounding probe deposition, sample preparation/delivery and read-out principles will also have to be addressed. In fact, these critical points have already attracted the attention of investigators. Second, high-density antibody microarrays require access to large numbers of antibody probes. Today, the only foreseeable option is to work with recombinant antibody libraries (that can contain >10^{10} preformed antibodies), where the probes must be handled without any purification steps. The reason for the latter resides in the fact that the logistics behind purifying 10,000 probes are daunting. This particular issue can be solved by on-chip affinity approaches using pretagged antibody fragments (i.e., the antibodies are carrying a genetically linked tag). However, many available affinity tags display binding affinities that are too low, thereby reducing the efficiency of coupling. Today, an attractive alternative is the double-His tag, fulfilling the requirements outlined above [STEINHAUER ET AL, UNPUBLISHED OBSERVATIONS]. Third, the term probe specificity is currently no longer relevant. These highly dense arrays would instead be analyzed based on pattern recognition, using, for example, artificial neural networks [47]. In the case of differential proteome analysis, patterns that differ between healthy versus diseased samples would first be analyzed and identified using the full arrays. Considering these observations, smaller and more focused microarrays could then be designed, based on perhaps less than 250 antibodies, to analyze and characterize the observed differences in more detail. Fourth, even though fluorescent labels are performing well, read-out systems based on the molecular interaction between an antibody and the corresponding antigen need to be developed. This would facilitate the array design and more importantly avoid any biased introduced during the labeling step. The latter point most likely poses the greatest challenge, since progress in soft lithography and affinity tags point to solutions not present 1–2 years ago, while massive protein–protein interactions are still difficult to measure with high sensitivity. The final product will then allow us to perform high-throughput, comparative proteomics with a resolution that no technology is even close to today. Furthermore, when coupled to MS, the antibody microarrays will also be useful for protein network studies, where the identity of the interacting molecules can rapidly be revealed adding another dimension to the microarrays.

Five-year view

Within the next few years, antibody microarray technology will become established as a high-throughput proteomic research tool. Engineered recombinant antibody fragments, designed for microarray applications, with predetermined on-chip characteristics, will replace mono- and polyclonal antibodies as the desired probe. The repertoire of novel, inert and highly biocompatible solid supports designed for affinity coupling of the content will increase. Furthermore, more focus will primarily be placed upon sample handling, in a manner similar to that which has been achieved within traditional proteomics. Also, platforms allowing water-soluble as well as membrane proteins to be targeted will become available. Efforts to further miniaturize the array format will be intensified, and the first semi-dense nanoarrays based on nanotechnology approaches will be in place. Although fluorescence will continue to be the dominat analytical principle, the selection of label-free read-out systems will increase. In addition, more direct approaches allowing the bound analytes to be characterized (e.g., binding kinetics, molecular weight) and even identified (amino acid sequence) directly on the array will be strongly favored.

Most strikingly, the number of applications will increase significantly. As low- to medium-density arrays will continue to be the dominating array format (at least for the commercially available array platforms), the focus will remain mainly within diagnostics and protein expression profiling. However, a clear focus towards disease proteomics will continue where cancer, autoimmunity and allergy will be at the forefront. In select

cases, combined efforts, adopting both DNA microarrays and antibody-based microarrays, to enable true global profiling at the gene as well as the protein level will be more commonly adopted. Taken together, antibody microarrays will evolve into a versatile proteomic research tool commonly used within academic, clinical and industrial settings.

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Key issues

- Antibody-based microarrays are a novel technology that hold great promise within the field of proteomics.
- Engineered recombinant antibody libraries designed for microarray applications are the preferred high-performance probe source of the future.
- To date, applications have been developed mainly within the areas of diagnostics, screening and protein expression profiling/focused proteome analysis.
- Current antibody microarray platforms are capable of performing rapid and highly multiplexed (focused) proteome analysis also targeting low-abundant protein analytes.
- Global proteome analysis and protein expression profiling using antibody microarrays will provide unique opportunities for biomarker discovery, drug target identification, disease diagnostics and insights into disease biology.
- High-throughput proteomics of clinically relevant samples, using antibody-based microarrays, will generate vast amounts of data that coalesce to form a molecular fingerprint of disease at the protein level.
- The full potential of antibody microarray technology remains to be explored and will be fully revealed when the remaining key technology issues have been fully addressed.

References

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1 Phizicky E, Bastiaens PIH, Zhu H, Snyder M, Fields S. Protein analysis on a proteomic scale. *Nature* 422(13), 208–215 (2004).

2 Staudt LM. Gene expression profiling of lymphoid malignacies. *Ann. Rev. Med.* 53, 303–318 (2002).

3 Macbeath G. Protein microarrays and proteomics. *Nature Genet.* 32, 526–532 (2002).

4 Zhu H, Bilgin M, Bangham R *et al.* Global analysis of protein activities using proteome chips. *Science* 293(14), 2101–2105 (2001).

•• **First global proteome chip reported, as well as novel data based on this chip design.**

5 Pavlickova P, Schneider EM, Hug H. Advances in recombinant antibody microarrays. *Clin. Chim. Acta.* 343(1–2), 17–35 (2004).

6 Borrebaeck CAK. Antibodies in diagnostics – from immunoassays to protein chips. *Immunol. Today* 21(8), 379–382 (2000).

7 Zhu H, Snyder M. Protein chip technology. *Curr. Opin. Chem. Biol.* 7(1), 55–63 (2003).

8 Wilson DS, Nock S. Recent developments in protein microarray technology. *Angew. Chem.* 42(5), 494–500 (2003).

9 Haab BB. Methods and applications of antibody microarrays in cancer research. *Proteomics* 3(11), 2116–2122 (2003).

10 Wingren C, Ingvarsson J, Lindstedt M, Borrebaeck CAK. Recombinant antibody microarrays – a viable option? *Nature Biotechnol.* 21(3), 223 (2003).

• **Discusses the issue of probe specificity and demonstrates the utility of recombinant antibody fragments as high-performance probes.**

11 Miller JC, Zhou H, Kwekel J *et al.* Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics* 3(1), 56–63 (2003).

• **First attempt to demonstrate comparative proteomics using antibody microarrays.**

12 Knezevic V, Leethanakul C, Bichsel VE *et al.* Proteomic profiling of the cancer microenvironment by antibody arrays. *Proteomics* 1(10), 1271–1278 (2001).

• **Reports one of the first applications for antibody microarrays within cancer research.**

13 Sreekumar A, Nyati MK, Varambally S *et al.* Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. *Cancer Res.* 61(20), 7585–7593 (2001).

• **Reports on one of the first applications for antibody microarrays within cancer research.**

14 Belov L, de la Vega O, dos Remedios CG, Mulligan SP, Christopherson RI. Immunophenotyping of leukemias using a cluster of differentiation antibody microarray. *Cancer Res.* 61(11), 4483–4489 (2001).

• **Demonstrates the first applications of antibody microarrays for phenotyping leukemias.**

15 Belov L, Huang P, Barber N, Mulligan SP, Christopherson RI. Identification of repertoires of surface antigens on leukemias using an antibody microarray. *Proteomics* 3(11), 2147–2154 (2003).

16 Hanash S. Disease proteomics. *Nature* 13(422), 226–232 (2003).

17 Zhu H, Bilgin M, Snyder M. Proteomics. *Ann. Rev. Biochem.* 72, 783–812 (2003).

18 Tirumalai RS, Chan KC, Prieto DA *et al.* Characterisation of the low molecular weight human serum proteome. *Mol. Cell. Proteomics* 2(10), 1096–1103 (2003).

19 Adkins JN, Varnum SM, Auberry KJ *et al.* Toward a human blood serum proteome. *Mol. Cell. Proteomics* 1(12), 947–955 (2002).

20 Pieper R, Gatlin C, Makusky AJ *et al.* The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics* 3(7), 1345–1364 (2003).

21 Anderson NL, Polanski M, Pieper R *et al.* The human plasma proteome: a non-redundant list developed by combination of four separate sources. *Mol. Cell. Proteomics* 3(4), 311–326 (2004).

22 Kingsmore SF, Patel DD. Multiplexed protein profiling in antibody-based microarrays by rolling circle amplification. *Curr. Opin. Biotechnol.* 14 (1), 74–81 (2003).

23 Mitchell P. A perspective on protein microarrays. *Nature Biotechnol.* 20(3), 225–229 (2002).

24 Service RF. Searching for recipes for protein chips. *Science* 294(5549), 2080–2082 (2001).

25 Haab BB, Dunham MJ, Brown PO. Protein microarrays for highly parallel detection and quantification of specific proteins and antibodies in complex solutions. *Genome Biol.* 2(2), 1–22 (2001).

• **Reports one of the first antibody microarray set-ups.**

26 Macbeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science* 289(585), 1760–1763 (2000).

• **Reports one of the first antibody microarray set-ups.**

27 Kusnezow W, Hoheisel JD. Antibody microarrays: promises and problems. *Biotechniques* 33(Suppl.), 14–23 (2002).

28 Steinhauer C, Wingren C, Malmborg-Hager A, Borrebaeck CAK. Single framework recombinant antibody fragments designed for protein chip applications. *Biotechniques* 33(Suppl.), 38–45 (2002).

29 Wingren C, Steinhauer C, Ingvarsson J, Persson E, Larsson K, Borrebaeck CAK. Microarrays based on affinity-tagged scFv antibodies: sensitive detection of analyte in complex proteomes. *Proteomics* (2004) (In Press).

30 Söderlind E, Strandberg L, Jirholt P *et al.* Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nature Biotechnol.* 18(8), 852–856 (2000).

31 Knappik A, Ge L, Honegger A *et al.* Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular concensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* 296(1), 57–86 (2000).

32 Ewert S, Huber T, Honegger A, Pluckthun A. Biophysical properties of human antibody variable domains. *J. Mol. Biol.* 325(3), 531–553 (2003).

33 Kusnezow W, Hoheisel JD. Solid supports for microarray immunoassays. *J. Mol. Recognit.* 16(4), 165–176 (2003).

34 Angenendt P, Glokler J, Sobek J, Lehrach H, Cahill DJ. Next generation of protein microarray support materials: evaluation for protein and antibody microarray applications. *J. Chromatogr. A* 1009(1–2), 97–104 (2003).

35 Angenendt P, Glokler J, Murphy D, Lehrach H, Cahill DJ. Toward optimized antibody microarrays: a comparison of current microarray support materials. *Anal. Biochem.* 309(2), 253–260 (2002).

36 Ressine A, Ekström S, Marko-Varga G, Laurell T. Macro-/nanoporous silicon as a support for high-performance protein microarrays. *Anal. Chem.* 75(24), 6968–6974 (2003).

37 Steinhauer C, Ressine A, Marko-Varga G, Laurell T, Borrebaeck CAK, Wingren C. Biocompatability of surfaces for microarrays based on recombinant antibody fragments. *Anal. Biochem.* (2004) (In Press).

38 Stillman BA, Tonkinson JL. FAST slides: a novel surface for microarrays. *Biotechniques* 29(3), 630–635 (2000).

39 Beator J. From protein microarrays to cytokine detection chips. *Biotech. International* 20–22 (2002).

40 Wang CC, Huang RP, Sommer M *et al.* Array-based multiplexed screening and quantitation of human cytokines and chemokines. *J. Proteome Res.* 1(4), 337–343 (2002).

41 Rubina AY, Dementieva EI, Stomakhin AA *et al.* Hydrogel-based protein microchips: manufacturing, properties, and applications. *Biotechniques* 34(5), 1008–1014, 1016–1020, 1022 (2003).

42 Kiyonaka S, Sada K, Yoshimura I, Shinkai S, Kato N, Hamachi I. Semi-wet peptide/protein array using supramolecular hydrogel. *Nature Mater.* 3(1), 58–64 (2004).

43 Svedhem S, Pfeiffer I, Larsson C, Wingren C, Borrebaeck CAK, Höök F. Patterns of DNA-labeled and protein/scFv-carrying lipid vesicles directed by preferential protein adsorption and supported lipid bilayer formation on an Au/SiO^2 template. *Chem. Biol. Chem.* 4(4), 339–343 (2003).

44 Peluso P, Wilson DS, Do D *et al.* Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal. Biochem.* 312(2), 113–124 (2003).

45 Pavlickova P, Knappik A, Kambhampati D, Ortigao F, Hug H. Microarray of recombinant antibodies using a streptavidin sensor self-assembled onto a gold layer. *Biotechniques* 34(1), 124–130 (2003).

46 Ruiz-Taylor LA, Martin TL, Zaugg FG *et al.* Monolayers of derivatized poly (L-lysine)-grafted poly(ethyleneglycol) on metal oxides as a class of biomolecular interfaces. *Proc. Natl Acad. Sci. USA* 98(3), 852–857 (2001).

47 Wingren C, Montelius L, Borrebaeck CAK. Mega-dense nanoarrays – the challenge of novel antibody array formats. In: *Protein Microarrays*. Schena M, Weaver S (Eds), Jones and Bartlett Publishers, MA, USA Chapter 17 (2004).

48 Hoff JD, Cheng LJ, Meyhöfer E, Guo LJ, Hunt AJ. Nanoscale protein patterning by imprint litography. *Nanoletters* (2004) (In Press).

49 Heidari B, Maximov I, Sarwe EL, Montelius L. Large scale nanolitography using nanoimprint litography. *J. Vac. Sci. Techn. B* 17, 2961–2964 (1999).

50 Demers LM, Ginger DS, Park SJ, Li Z, Chung SW, Mirkin CA. Direct patterning of modified oligonucleotides on metals and insulators by dip-pen litography. *Science* 296(5574), 1836–1838 (2002).

51 Bruckbauer A, Zhou D, Kang DJ *et al.* An addressable antibody nanoarray produced on a nanostructured surface. *J. Am. Chem. Soc.* 126(21), 6508–6509 (2004).

52 Moody MD, Van Arsdell SW, Murphy KP, Orencole SF, Burns C. Array-based ELISAs for high-throughput analysis of human cytokines. *Biotechniques* 31(1), 393–397 (2000).

53 Ekins RP. Ligand assays: from *electrophoresis* to miniaturized microarrays. *Clin. Chem.* 44(9), 2015–2030 (1998).

54 Pawlak M, Schick E, Bopp MA, Schneider MJ, Oroszlan P, Ehrat M. Zeptosens's protein microarrays: a novel high performance microarray platform for low abundance protein analysis. *Proteomics* 2(4), 383–393 (2003).

55 Schweitzer B, Wiltshire S, Lambert J *et al.* Immunoassays with rolling circle DNA amplification: a versatile platform for ultrasensitive antigen detection. *Proc. Natl Acad. Sci. USA* 97(18), 10113–10119, (2000).

56 Schweitzer B, Roberts S, Grimwade B *et al.* Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nature Biotechnol.* 20(4), 359–365 (2002).

57 Zhou H, Bouwman K, Schotanus M *et al.* Two-color, rolling circle amplification on antibody microarrays for sensitive multiplexed serum-protein measurements. *Genome Biol.* 5(4), R28 (2004).

58 Borrebaeck CAK, EkströmS, Malmborg-Hager AC, Nilsson J, Laurell T, Marko-Varga G. Protein chips based on recombinant antibody fragments: a highly sensitive approach as detected by mass spectrometry. *Biotechniques* 30(5), 1126–1132 (2001).

59 Larsson C, Bramfeldt H, Wingren C, Borrebaeck Cak, Höök F. Coupling of his-tagged scFvs from expression supernatants to NTA/Ni^{2+}-functionalized supported lipid bilayers for specific antigen detection using QCM-D. (2004) (In Press).

60 Arntz Y, Seelig JD, Lang HP *et al.* Label-free protein assay based on a nanomechanical cantilever array. *Nanotechnol.* 14, 86–90 (2003).

61 Dutta P, Tipple CA, Lavrik NV *et al.* Enantioselective sensors based on antibody-mediated nanomechanics. *Anal. Chem.* 75(10), 2342–2348 (2003).

62 Huang RP, Huang R, Fan Y, Lin Y. Simultaneous detection of multiple cytokines from conditioned media and patient's sera by an antibody-based protein array system. *Anal. Biochem.* 294(1), 55–62 (2001).

63 Huang RP. Cytokine protein arrays. *Methods Mol. Biol.* 264, 215–231 (2004).

64 Lin Y, Huang RP, Chen LP *et al.* Profiling of cytokine expression by biotin-labeled based protein arrays. *Proteomics* 3(9), 1750–1757 (2003).

65 Sreekumar A, Chinnaiyan AM. Using protein microarrays to study cancer. *Biotechniques* 33(Suppl.), 46–53 (2002).

66 Harwanegg C, Hiller R. Protein microarrays in diagnosing IgE-mediated diseases: spotting allergy at the molecular level. *Expert Rev. Mol. Diagn.* 4(4), 539–548 (2004).

Websites

101 Affibody
www.affibody.com
(Viewed September 2004)

102 SomaLogic
www.somalogic.com
(Viewed September 2004)

103 Aspira Biosystems
www.aspirabio.com
(Viewed September 2004)

104 Archemix
www.archemix.com
(Viewed September 2004)

105 Phylos
www.phylos.com
(Viewed September 2004)

106 Bioinvent
www.bioinvent.se
(Viewed September 2004)

107 Cambridge Antibody Technology
www.cambridgeantibody.com
(Viewed September 2004)

108 PerkimElmer
www.perkinelmer.com
(Viewed September 2004)

109 Scandinavian Micro Biodevices
www.smb.dk
(Viewed September 2004)

110 Xenopore
www.xenopore.com
(Viewed September 2004)

111 Schleicher & Schuell
www.schleicher-schuell.com
(Viewed September 2004)

112 Nunc
www.nuncbrand.com
(Viewed September 2004)

113 TeleChem International, Inc.
www.arrayit.com
(Viewed September 2004)

114 Ciphergen
www.ciphergen.com
(Viewed September 2004)

115 Biosite
www.biosite.com
(Viewed September 2004)

116 MorphoSys
www.morphosys.com
(Viewed September 2004)

117 Zyomyx
www.zyomyx.com
(Viewed September 2004)

118 RayBiotech, Inc.
www.raybiotech.com
(Viewed September 2004)

119 EMD Biosciences
www.emdbiosciences.com
(Viewed September 2004)

120 Molecular Staging Inc.
www.molecularstaging.com
(Viewed September 2004)

121 Zeptosens
www.zeptosens.com
(Viewed September 2004)

122 BD Biosciences
www.bdbiosciences.com
(Viewed September 2004)

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